Phylogenetic affinities and conservation status of the Chilean endemic *Costesia spongiosa* (Gigaspermaceae)

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Abstract. *Costesia spongiosa* is known only from central Chile. The recent discovery of several small populations prompted an examination of diagnostic morphological features and the sampling of DNA for surveying the chloroplast genome for a 71-kb inversion diagnostic of the Funariales and Encalyptales, and for reconstructing the phylogenetic affinities of this monospecific genus. The species is fully illustrated and mapped. The affinities of *Costesia* to the Gigaspermaceae are here confirmed based on morphology and corroborated by chloroplast sequence data. Molecular data suggest that *Costesia* is most closely related to *Oedipodiella* and *Chamaebryum*. Although the species is locally common, it has disappeared from several localities and seems now confined mostly to a protected area. We propose that *Costesia spongiosa* be included as the first Chilean endemic bryophyte in the IUCN Red List of Threatened Species.

Keywords. *Costesia*, Gigaspermaceae, Funariidae, Chile, La Campana National Park, *trn*L-F, *rps*4, phylogeny, SEM.

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In 1865 Lindberg established *Gigaspermum* and the family Gigaspermaceae to accommodate a single member of the Hedwigian pottiaceous genus *Anictangium* (*nom. rejic.*, later corrected by Schwägrichen [1811] to *Anoectangium*). Unlike other members of *Anoectangium*, *A. repens* [= *Gigaspermum repens*] produces very large spores, has smooth laminal cells, a rhizome, and erect branches. The circumscription of the family was subsequently expanded to include a) *Lorentziella* known from Argentina, Uruguay (Müller 1879, 1888), Texas (Lawton 1953) and Mexico (Cárdenas & Delgadillo 1994); b) *Chamaebryum* from South Africa (Dixon 1922); and c) *Oedipodiella* from South Africa (Dixon 1922) and Europe (Brugués 2003). As part of his generic revision of the Funariaceae, Fife (1980) examined the affinities of *Neosharpiella* from Latin America and *Costesia* from central Chile. He concluded that both genera should be transferred to the Gigaspermaceae.

Thériot (1917) described *Costesia* based on material collected in 1915 by the French priest Nathaniel Costes in Viña del Mar and in Los Perales de Marga-Marga (Region V, Valparaíso Province).

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Unambiguous type material has not yet been located, but the specimens at CONC that Thériot had annotated, along with the description and illustrations given by Thériot (1917), offer the necessary references for identifying material.

In his checklist of Chilean mosses, He (1998) omitted Fife's systematic change and kept *Costesia* in the Funariaceae. He also omitted Fife's report of *Neosharpiella turgida*, based on a single collection by L. Landrum in San Clemente, Talca (*Landrum 302*, COLO, CONC, SGO), thus not including the Gigaspermaceae in the moss flora of Chile.

The Gigaspermaceae have traditionally been considered allied to the Funariaceae, within the Funariales (Vitt 1984). The two families differ in a number of morphological traits (Brotherus 1924; Fife 1980). Recent phylogenetic evidence suggests that the Funariaceae and Gigaspermaceae share a common ancestor with the Encalyptaceae (Goffinet & Cox 2000). Surprisingly, support for the monophyly of the Funariales was lacking, and the Funariaceae appeared most closely related to the Encalyptaceae. This hypothesis is congruent with the distribution of a 71-kb inversion in the large single-copy region of the plastid genome: the inversion occurs in the Funariaceae and Encalyptaceae, but is lacking in the Gigaspermaceae, which were thereupon accommodated in their own order (Goffinet et al. 2007).

The recent rediscovery of *Costesia spongiosa* in central Chile nearly 30 years after the last known collection, made in the Valparaíso region by professor Manuel Mahú in 1981, prompted us to reëxamine the morphological characteristics of the species, assess its phylogenetic affinities based on DNA sequence data, and summarize its geographical and historical distribution to estimate its conservation needs. Our observations confirm the inclusion of *Costesia* within the Gigaspermaceae and reveal a sister-group relationship with *Chamaebryum* and *Oedipodiella*. Further, our observations indicate that *Costesia spongiosa* is endemic to central Chile where it is exceedingly rare, and known from few extant populations.

Materials and Methods

Plant material. Collections of *Costesia spongiosa* kept at COLO, CONC, CONN, MO, PC, S and SGO were studied. Additionally, several fresh specimens were collected by the senior author since 2005 in La Campana National Park (Quillota Province, Region V, central Chile) and surrounding areas.

DNA extraction, amplification and sequencing. DNA was extracted from a dried herbarium specimen of *Costesia spongiosa* (Chile, Region V, Quillota Province, Cerro La Campana, *Larraín & Zegers 27172*, CONN) using a standard CTAB protocol (Murray & Thompson 1980). Previously, DNA was extracted from *Oedipodiella australis* (France, Pyrenees-Orientales, Commune d'Argeles, sur Mer. Vallee du Rouvaner, 2005, *Thouvenot s.n.*, CONN) by Goffinet et al. (2007). We used the PCR approach outlined in Goffinet et al. (2007; primer pairs trnC-Fun/rpoBR-2 and Giga-petD-R2/rps11F) to determine whether the chloroplast genome of *Costesia* includes the 71-kb inversion.

The chloroplast regions *trn*L-*trn*F and *rps*4 were targeted with the same primers as Werner et al. (2007). PCRs included 2.5 µl of HotMaster *Taq* Buffer (Eppendorf), 0.75 units HotMaster *Taq* DNA Polymerase (Eppendorf), 2.5 mM of each dNTP, 1.0 µl of each primer at 10 mM, and 1.0 µl of genomic DNA in a total reaction volume of 25 µl. Reactions were carried out on a MJ Research 220 Peltier Thermal Cycler using the following conditions: 1.5 min at 94°C, followed by 30 cycles of 20 s at 95°C, 45 s at 52°C and 1 min at 68°C. The reaction was terminated by a final extension of 7 min at 68°C. PCR products were cleaned using a Nucleospin purification kit (BD Biosciences).

Sequencing reactions contained: 0.5 µl of purified PCR product, 1.0 µl of one primer used in the PCRs, and 2.0 µl of BigDye Terminator v1.1 (Applied Biosystems) in a total reaction volume of 10 µl. These reactions used the following conditions: 2 min at 96°C, followed by 25 cycles of 30 s at 96°C, 15 s at 50°C and 4 min at 60°C. Sequencing reaction products were purified using Sephadex columns (Amersham Biosciences). Sequences were determined using an ABI PRISM 3100 (Applied Biosystems) automated sequencer.

Sequences were manually aligned with the matrix from Werner et al. (2007) and ambiguously aligned regions were excluded. Phylogenetic analyses were carried out using the criteria of maximum parsimony

(MP), maximum likelihood (ML) and Bayesian inference. The search strategies and references were identical to those of Werner et al. (2007) unless otherwise stated. MrModeltest (Nylander 2004) identified GTR+I+G as the optimal model using the Akaike Information Criterion (AIC). The following settings were used: base frequencies (A:0.4252, C:0.1220, G:0.1301 & T:0.3227), relative substitution rates (A–C: 1.0607, A–G: 4.8276, A–T: 0.3005, C–G: 1.5651, C–T: 4.9735 and G–T: 1.0000), proportion of invariable sites (I = 0.2347) and gamma distribution shape parameter ($\gamma = 0.6531$). Branch support for ML trees was calculated using the program GARLIv0.9.5.1 (www.bio.utexas.edu/faculty/antisense/garli/Garli.html; Zwickl 2006). This bootstrap analysis was performed using default conditions with 100 pseudoreplicates. Bootstrap proportions were obtained from the 70% majority-rule consensus tree of the optimal MP or ML trees. In the Bayesian analyses, the first 1000 trees saved during each of the four runs were excluded. The remaining 76,000 trees were combined and the posterior probability values obtained from the 95% majority-rule consensus tree.

SEM photographs. Dried plants were hydrated in distilled water and then fixed in glutaraldehide 2.5% in sodium cacodilate buffer 0.1 M (pH 7.2–7.4) for 24 hours at 4°C. Then the material was dehydrated in an acetone ladder, through six ascending steps starting at 30% acetone until reaching 100%. The material was then subjected to critical point drying in liquid CO_2 following the protocol in Anderson (1951). The material was then fixed on a metal plate and covered with a gold layer. The plants were observed and photographed using a JEOL JSM-6380 LV scanning electron microscope.

Results and Discussion

Costesia spongiosa Thér., Revista Chil. Hist. Nat. 21: 12, fig. 3. 1917. Type: Chile. Viña del Mar, Aug 1915; Los Perales de Marga-Marga, Jun 1915, sur la terre. Leg. *N. Costes* (unambiguous type material not found). **Figs. 1–3**

Description. Plants small, delicate, gregarious or forming small groups, whitish-green to dark dull green, becoming darker in old herbarium specimens. Stems dimorphic: primary stems prostrate, sympodially branched, subterranean, whitish, fragile, variable in length, reaching 10 mm long or more, in cross-section rounded, 0.3–0.4 mm in diameter, formed by a lax cortex of thin-walled cells, 50–60 μm in diam., with a scarcely differentiated epidermis, central strand lacking, bearing a dense tomentum of hyaline to brownish-hyaline smooth rhizoids to 10 mm long; secondary stems erect, unbranched, arising from the subterranean primary stems, 1–3 mm long, without leaves in lower part, with the same anatomy as that of the prostrate stems, bearing fewer rhizoids; axillary hairs not seen. Leaves rosulate, plane to slightly concave, becoming larger in the upper part of stems, spathulate to oblong, acuminate, widest at or above the middle, (2.0−)3.0−3.5 × (1.0−)1.2−1.5 mm, unbordered; margins entire to rarely uneven in apex of old leaves, plane; costa single, strong, subpercurrent, 150–220 μm wide at base, gradually tapering towards the apex, vanishing (2−)4−8(−10) cells below the apex, dorsally convex and rounded in cross-section, surrounded by a layer of rectangular (3–8 times as long as wide) epidermal cells enclosing smaller cells; laminal cell walls thin and lax, with slightly thickened corners, straight in the lower cells, becoming wavy in central cells, uneven and irregular in upper part of the leaf; basal cells rectangular, 3–5 times as long as wide; upper cells quadrate to irregularly shaped and more or less isodiametric; alar cells not differentiated. Synoicous. Antheridia 6–10 per inflorescence, stalked; archegonia 4–8; paraphyses 5–10, smooth, 8–12 cells long, the terminal cells much shorter and the most distal not or only scarcely inflated. Perichaetial leaves much smaller than the vegetative leaves, 1.0−1.2 × 0.7−0.9 mm, ovate, acuminate. Seta sinistrose, 2.5–5.0 mm long, yellowish to reddish brown, epidermis with thick outer walls; central strand present. Capsule globose to oval, wrinkled when dry, 1.5−2.5 × 1.0−1.5 mm, brown at maturity; operculum shortly conic-apiculate when wet, becoming flat and mammillate when dry; annulus conspicuous, of 2–3 rows of very small oblate cells over 3–4 rows of isodiametric, irregularly shaped and thick-walled cells beneath them; exothecial cells thin-walled, hexagonal to irregularly shaped; stomata abundant in the base of the capsules, superficial, 20–30, surrounded by two guard cells or sometimes appearing as one single split cell;

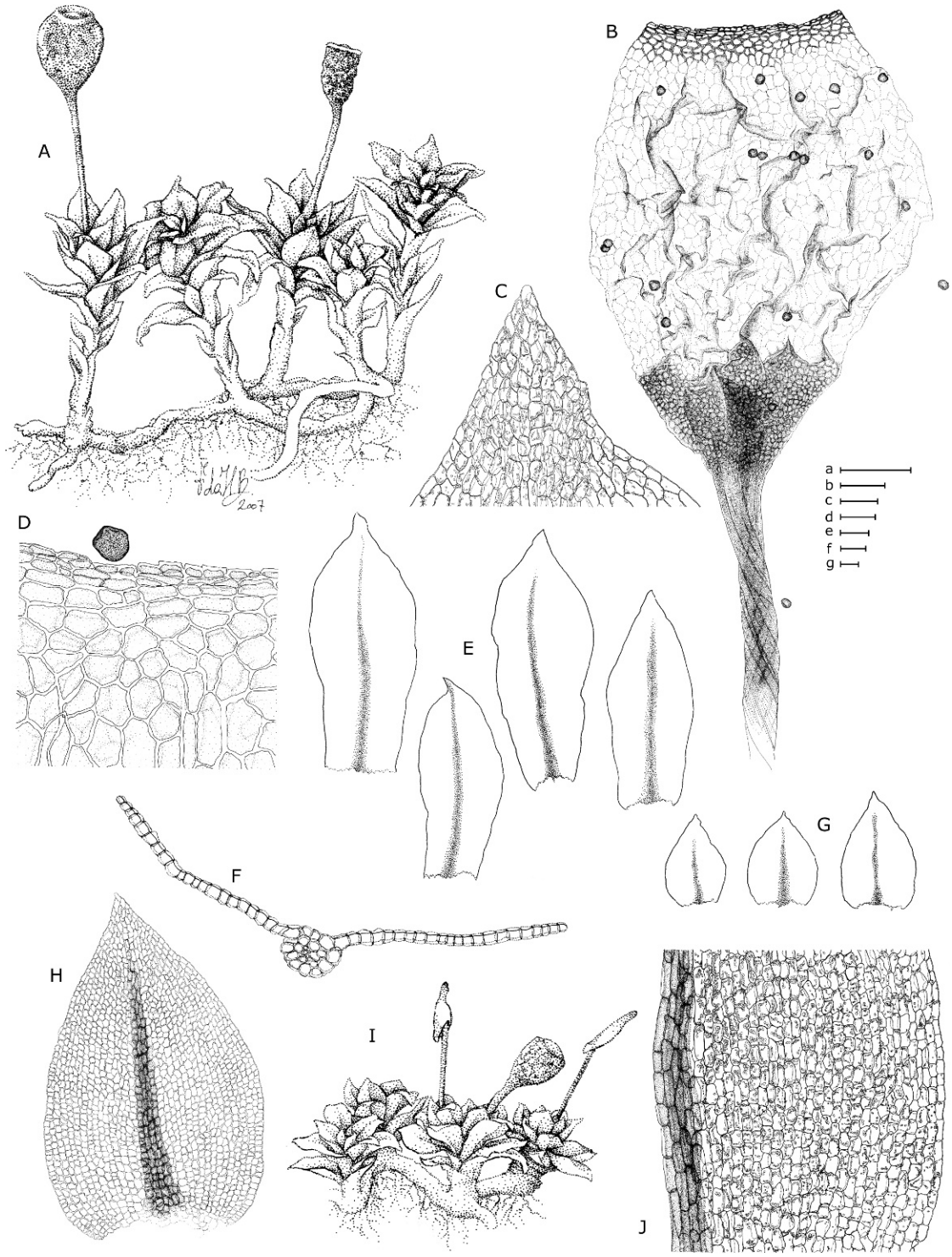

Figure 1. *Costesia spongiosa*. **A.** Habit, wet. **B.** Capsule, wet. **C.** Cells of leaf apex. **D.** Portion of capsule mouth. **E.** Leaves. **F.** Leaf cross section at midleaf. **G.** Perichaetial leaves. **H.** Areolation of perichaetial leaf. **I.** Plants with sporophytes bearing young calyptrae. **J.** Leaf areolation showing costa and adjacent laminal cells at midleaf. (A, E–G, J drawn from *Larraín 26086*, CONC; B, D, H drawn from *Larraín 26085*, CONC; C drawn from *Larraín 26087*, CONC; I drawn from *Larraín 26084*, CONC). Scale bars: a = 2 mm (A, I); b = 0,5 mm (E, G) and 100 µm (F); c = 50 µm (D); d = 100 µm (J); e = 50 µm (C); f = 100 µm (H); g = 100 µm (B).

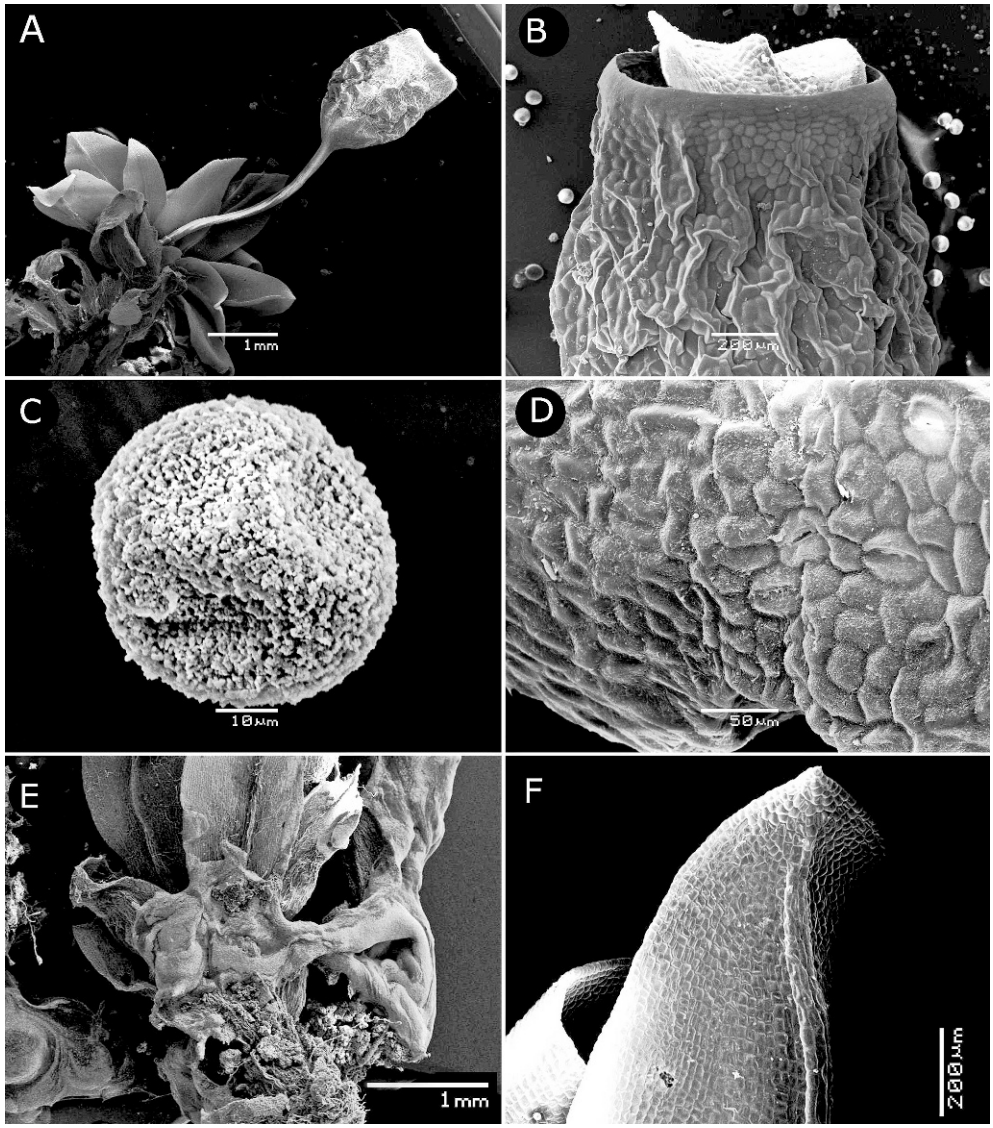

Figure 2. *Costesia spongiosa*. SEM photographs. **A.** Habit, dry. **B.** Capsule showing detached operculum. **C.** Spore. **D.** Base of capsule showing stomata. **E.** Subterranean stem. **F.** Leaf apex (all from *Larraín 26084*, CONC)

peristome absent. Spores large, 42–58 µm in diameter, densely verrucate to baculate, brown. Calyptra cucullate, small, deciduous, 1.7–1.8 mm long, hyaline with a reddish-brown tip.

Specimens examined. CHILE. REGION IV. CHOAPA: Pichidangui, Nov 1976, *Weber & Johnston B-58009* (COLO, SGO); REGION V. QUILLOTA: Cerro La Campana, *Larraín & Zegers 27172* (CONC, CONN, S); Campanino, Oasis de La Campana, *Larraín 26084* (CONC, CONN, S), *Larraín 25057*, *25062 A*, *25064*, *26085*, *26086*, *26087* (CONC), May 2008, *Bellolio & Ireland 36450* (CONC); Parque Nacional La Campana, sector Ocoa, Aug 1977, *Mahú 11361* (CONC, MO), Sep 1977, *Mahú 23816* (MO), *Larraín & Vargas 29600*, *29602* (CONC), May 2008, *Bellolio & Ireland 36458* (CONC); Cuesta Las Chilcas, Jun 2003, *García s.n.* (CONC), *Larraín et al. 31401*, *31405* (CONC), Estero Los Loros, *s.col.* (CONC); VALPARAíSO: collines de Playa Ancha, Jun 1945, *Jaffuel s.n.* (PC 94839); Los Perales de Marga-Marga, Nov 1919, *Jaffuel s.n.* (CONC, S); entre Quilpué y Lo Vasquez, Aug 1981, *Mahú 13687* (MO); El Quisco, Punta de Tralca, Feb 1976, *Mahú 10664* (MO); REGION VI. CACHAPOAL: Pelequén, Aug 1896, *Dusén 166* (S).

Figure 3. *Costesia spongiosa*. Field photograph of fresh plants at La Campana National Park, Quillota Province, Región V (*Larraín & Vargas 29600*, 17 Sep 2007, CONC)

Morphological traits. Most of the characters given by Fife (1980) for placing *Costesia* in the Gigaspermaceae are confirmed by light (**Fig. 1**) and scanning electron microscopy (**Fig. 2**). The fragile whitish subterranean rhizomes that give rise to erect branches, the non-inflated and cucullate calyptra, the stomata enclosed by two guard cells (although sometimes they appear as just one single split cell), paraphyses with non-inflated terminal cell, and the large size of the spores confirm its placement in the family. The spore ornamentation (**Fig. 2C**) is similar to that of *Oedipodiella australis* (Fife 1980, fig. 3), and very different from the ornamentation of *Neosharpiella*, *Lorentziella* or *Gigaspermum* spores, as shown by Fife (1980, figs. 1, 2 and 4). In fact the ornamentation very much resembles the *Funaria* baculate-type, as defined by Hirohama (1978, figs. 31, 32), although the spore size is much smaller in *Funaria*. Thériot's (1917) original description of the operculum shape ("…*depressum, plano-convexum, maturitate breve mamillatum*"), a character not seen by Fife (1980), is confirmed (**Fig. 2B**). However, it is worth noting that the shape of the operculum varies from flat when dry (**Fig. 2B**) to sometimes almost conical in living plants (**Fig. 3**). Finally, the perichaetial leaves are small and ovate (**Fig. 1G, H**) compared to the vegetative leaves that are large and spathulate (**Fig. 1E**). In *Neosharpiella*, *Lorentziella* and *Gigaspermum* this trait is just the opposite: the perichaetial leaves are larger and more acuminate than the rest of the leaves.

The species has been collected in all four seasons of the year, even at the end of the dry season, when most of the plants look quite dry, but some green specimens are occasionaly observed. The old capsules remain for almost a year after new sporophytes are produced. This fact, together with the presence of apparently persistent primary subterranean stems, suggest that the species could be perennial, although a more careful study is needed to confirm this observation. Sporophytes are produced in the winter (Jun–Aug) and fresh mature capsules have been observed from Sep–Nov. The material studied shows all developmental stages, and it has been possible to observe all gametophytic and sporophytic traits on it.

Phylogenetic relationships. Targeting the areas spanning across the putative breaking points of the inversion yielded positive PCR results, suggesting that the chloroplast genome of *Costesia* lacks the inversion. The amplicons were sequenced (EU700312 and EU700313) and both could be aligned with their homologous regions in the Gigaspermaceae (results not shown). Phylogenetic inferences based on the two chloroplast loci sampled corroborated an affinity to the Gigaspermaceae. The aligned matrix of Werner et al. (2007) with the addition of the *trn*L-*trn*F and *rps*4 loci from *Costesia spongiosa* (GenBank numbers EU681957 and EU681956, respectively) and *Oedipodiella australis* (EU681958 and EU681955) comprised 1,402 sites, of which 546 were excluded. The remaining 856 characters included 236 that are potentially parsimony informative. The MP analysis produced four most parsimonious trees, each with a length of 946, a consistency index (CI) of 0.56 and a retention index (RI) of 0.72. The ML heuristic search resulted in six optimal trees with a score of -Ln = 6100.9673, one of which is displayed in **Fig. 4**. Inferences under the MP, ML and Bayesian

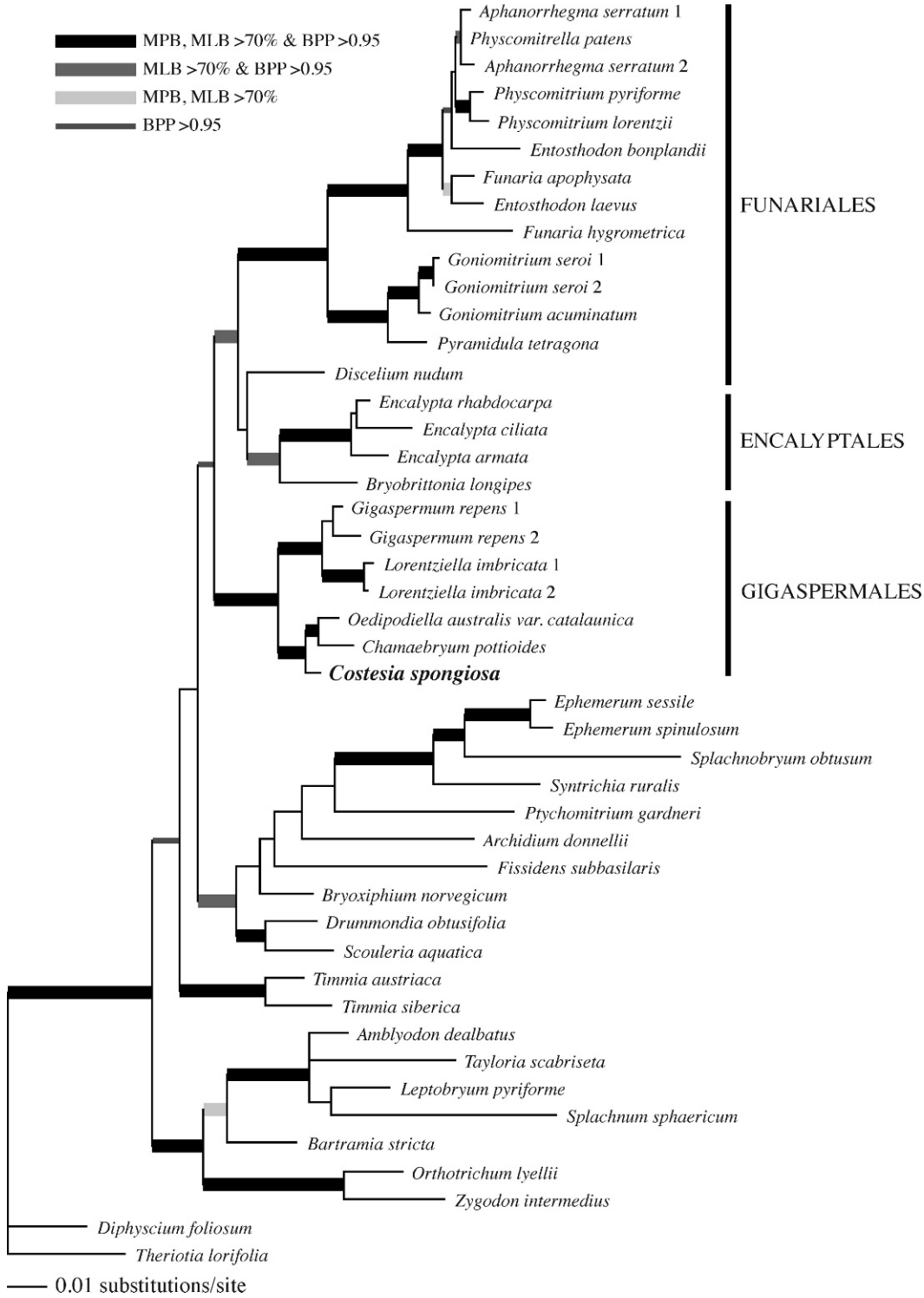

Figure 4. Phylogenetic affinities of *Costesia* within the Funariidae (Funariales, Encalyptales and Gigaspermales) based on *trn*L-*trn*F and *rps*4 sequences. Phylogram of one of the six trees with the highest likelihood value (-Ln = 6100.9673) from the ML analysis. Thickness and shading of branches indicates support values from Maximum Parsimony Bootstrap (MPB) >70%, Maximum Likelihood Bootstrap (MLB) >70%, and Bayesian Posterior Probabilities (BPP) >0.95.

optimality criterion yielded congruent phylogenetic signals: alternative branching orders were not characterized by high support values as measured by bootstrap frequencies >70% or posterior probabilities >0.95.

The phylogenetic relationships among the Encalyptales, Funariales and Gigaspermales, or within the former two orders are not affected by the inclusion of *Costesia* and *Oedipodiella*: the monophyly of the Funariaceae remains well supported, whereas the inclusion of the Disceliaceae in the Funariales remains ambiguous (**Fig. 4**). Similarly the shared ancestry of all three orders (i.e., the monophyly of the Funariidae) lacks support in this analysis. *Oedipodiella*, represented by *O. australis*, is the sister-taxon to *Chamaebryum* (MPB=83%, MLB=85%, BPP=0.98). These two genera share a common ancestor with *C. spongiosa* and together they compose one well-supported (MPB=93%, MLB=89%, BPP=1.0) lineage in the Gigaspermales. The other lineage includes *Gigaspermum* and *Lorentziella* (MPB=99%, MLB=99%, BPP=1.0).

In terms of morphological affinities, all genera in the Gigaspermaceae share the presence of an apparently perennial, rhizome-like, prostrate primary stem, without central strand, that gives rise to erect branches bearing entire, somewhat concave leaves, with or without costa, becoming generally larger towards the apex of the branches; laminal cells smooth with thin walls generally thickened in the corners; synoicous or paroicous sexual condition; paraphyses filiform without a globose terminal cell; stomata superficial, formed by two guard cells or by one single split cell; capsules stegocarpous (cleistocarpous in *Lorentziella* and *Oedipodiella*) and gymnostomous; spores large (generally >50 μm); and calyptrae very small, cucullate and deciduous. Comparing morphological traits among the genera of Gigaspermaceae (Fife 1980) with the tree in **Fig. 4**, the clade formed by *Gigaspermum* and *Lorentziella* share the piliform leaf apex (acute to obtuse and mucronate in the other genera), the thick laminal cell walls (thin to firm in the rest), and the very large—over 100 μm in diameter—almost smooth spores (no larger than 65 μm and warted to verrucate in the other genera). The most problematic trait for

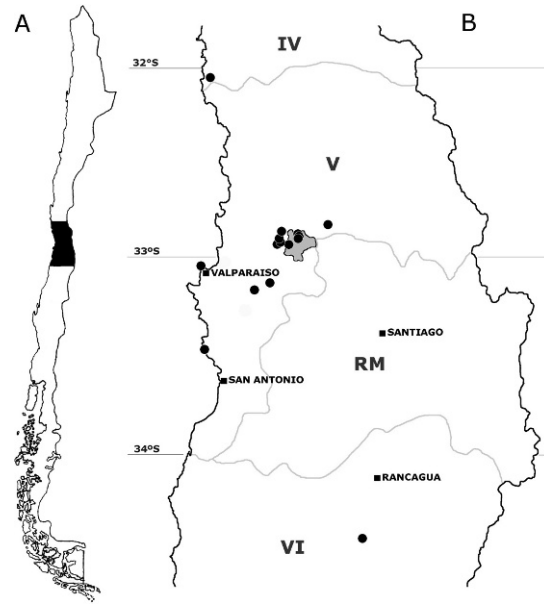

Figure 5. Distribution map of *Costesia spongiosa*. **A.** Map of Chile showing in black the central regions. **B.** Detailed map of the central regions of Chile showing observed specimens (black dots), and La Campana National Park (gray patch). (Roman numerals indicate Regions, RM being the Metropolitan Region)

defining these two separate clades within the Gigaspermace, as shown in **Fig. 4**, is the cleistocarpous capsules of *Lorentziella* and *Oedipodiella*, a character that should have evolved independently in the two taxa if one assumes the phylogeny given by the chloroplast *trn*L-*trn*F and *rps*4 sequences.

Geographic distribution. He (1998) reported *Costesia spongiosa* only from Quillota and Valparaíso provinces in Valparaíso Region (V) in central Chile. We discovered additional populations in Regions IV and VI, as well as in other localities in Region V, extending the known distribution of the species to the north and to the south (**Fig. 5**). The species has been observed growing from near the sea level to 800 m, where it grows on bare soil on the ground or on soil banks next to roads or creeks, in very dry conditions. *Costesia spongiosa* is a locally common species in La Campana National Park and adjacent areas to the west of the park, but it is very rare outside this protected area, where it is known just in seven other localities (**Fig. 5**). This may be explained by the strong and permanent anthropogenic disturbance this area has suffered, being by far the

most densely populated area in Chile, and affected by intense farming, grazing and associated habitat destruction. Some populations of the species in Regions IV (Pichidangui, Nov 1976, *Weber & Johnston B-58009*, COLO, SGO) and VI (Pelequén, Aug 1896, *Dusén 166*, S) are believed to be extinct since intense field work in central Chile made by the senior author has not yielded any other record in these areas. Human disturbance in both localities has been constant for more than 100 years, and very strong in the last 30 years. The few collections available of this taxon, together with the systematic singularity of the species and its narrow endemic distribution, require its protection. We propose the species should be considered in the IUCN Red List of Threatened Species as Vulnerable (VU B1a + B1b(i, ii, iii, iv) + B1c(ii)), following the criteria given in UICN (2001).

Acknowledgments

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